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## Interaction between $\gamma$ C87 and $\gamma$ R242 residues participates in energy coupling between catalysis and proton translocation in *Escherichia coli* ATP synthase

Yunxiang Li<sup>a,b,\*</sup>, Xinyou Ma<sup>c</sup>, Joachim Weber<sup>b,d</sup>

<sup>a</sup> Department of Chemistry and Biochemistry, Texas Woman's University, Denton, TX 76204 USA

<sup>b</sup> Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409 USA

<sup>c</sup> Department of Chemistry, The University of Chicago, Chicago, IL 60637 USA

<sup>d</sup> The Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX 79430 USA

## Abstract

Functioning as a nanomotor, ATP synthase plays a vital role in the cellular energy metabolism. Interactions at the rotor and stator interface are critical to the energy transmission in ATP synthase. From mutational studies, we found that the  $\gamma$ C87K mutation impairs energy coupling between proton translocation and nucleotide synthesis/hydrolysis. An additional glutamine mutation at  $\gamma$ R242 ( $\gamma$ R242Q) can restore efficient energy coupling to the  $\gamma$ C87K mutant. Arrhenius plots and molecular dynamics simulations suggest that an extra hydrogen bond could form between the side chains of  $\gamma$ C87K and  $\beta_{TP}E381$  in the  $\gamma$ C87K mutant, thus impeding the free rotation of the rotor complex. In the enzyme with  $\gamma$ C87K/ $\gamma$ R242Q double mutations, the polar moiety of  $\gamma$ R242Q side chain can form a hydrogen bond with  $\gamma$ C87K, so that the amine group in the side chain of  $\gamma$ C87K will not hydrogen-bond with  $\beta$ E381. As a conclusion, the intra-subunit interaction between positions  $\gamma$ C87 and  $\gamma$ R242 modulates the energy transmission in ATP synthase. This study should provide more information of residue interactions at the rotor and stator interface in order to further elucidate the energetic mechanism of ATP synthase.

Xinyou Ma: Software, Formal analysis, Investigation, Resources, Writing - review & editing.

<sup>&</sup>lt;sup>\*</sup>Corresponding author: Yunxiang Li, Ann Stuart Science Complex P.O. Box 425859. Department of Chemistry and Biochemistry, Texas Woman's University, Denton, TX 76204-5859 USA. Phone: (940) 898-2662; Fax: (940) 898-2548; yli7@twu.edu. Author contributions

Yunxiang Li: Conceptualization, Software, Validation, Formal analysis, Investigation, Project administration, Resources, Data curation, Writing - original draft, Writing - review & editing.

Joachim Weber: Conceptualization, Formal analysis, Resources, Writing - review & editing, Supervision, Funding acquisition. Conflict of interest

The authors declare that there is no conflict of interest.

E. coli numbering is used throughout this study.

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#### Keywords

ATP synthase; energy transmission; molecular dynamics; mutational study; residue interaction

#### 1. Introduction

F<sub>1</sub>Fo ATP synthase plays a critical role in the cellular energy metabolism. A functional ATP synthase in *E. coli* requires the proper assembly of eight types of subunits in a stoichiometry of  $ab_2c_{10}\alpha_3\beta_3\gamma\delta\epsilon$  (See Supplementary Material Fig. S1A and B for the quaternary structure of the holoenzyme and the terminology used throughout this article) [1]. Its unique rotary mechanism couples two distinct functions: proton translocation and ATP synthesis/ hydrolysis [2]. Considering that the binding locations of proton and nucleotide are approximately 100 Å apart [3], the central shaft  $\gamma$  subunit is essential to maintain the energy transmission in ATP synthase [4]. Protons flowing through two membrane half-channels in the a and c subunits push the rotor complex ( $c_{10}\gamma\epsilon$ ) to spin. The torque rotating the  $\gamma$  subunit within the  $\alpha_3\beta_3$  cylinder alters the conformation of the nucleotide binding pockets in the  $\beta$  subunits to accomplish ATP synthesis [5]. Each of these three  $\beta$  subunits adopts a different conformation depending on the rotation angle of the  $\gamma$  subunit [6]. According to the nucleotide occupancy in the original crystal structure, those three  $\beta$  subunits are named as  $\beta_{TP}$  (AMP-PNP bound),  $\beta_{dp}$  (ADP bound) and  $\beta_e$  (empty) [7].

Functional energy conversion and transmission in ATP synthase require proper interactions between  $\beta$  and  $\gamma$ . Uncoupling can occur when the energy flow is disrupted. ATP cannot be synthesized when the proton gradient is dissipated otherwise; or vice versa, a proton gradient cannot be formed by ATP hydrolysis [8]. The importance of several conserved residues located at the  $\beta$  and  $\gamma$  interface was discovered in mutational studies. For instance, *E. coli* ATP synthase with  $\beta$ E381K,  $\gamma$ S12A or  $\gamma$ M23K mutations is incapable to use carbon source efficiently *in vivo* [4,9,10]. Residue  $\gamma$ C87 is largely conserved, or occasionally replaced by alanine, but neither bulky nor charged amino are found in this position.  $\gamma R242$ in the C-terminus of the  $\gamma$  subunit is completely conserved (Supplementary Material Table S1). Previous studies have shown that *E. coli* ATP synthase with a  $\gamma$ C87A mutation mirrors the WT behaviors in microbial growth yield, ATPase activity and ATP-driven proton pumping ability [4]. The cysteine at  $\gamma$ C89 in spinach thylakoid ATP synthase (which is equivalent to  $\gamma$ C87 in *E. coli*) is a target of H2O2 oxidation; the  $\gamma$ C89A mutant can maintain high ATPase activity (85 % of the WT) [11]. E. coli ATP synthase with  $\gamma R242C$ mutation is WT-like, and it can restore the energy coupling caused by the  $\gamma$ M23K mutation [12]. With the  $\gamma$ R242E mutation in ATP synthase, no F1 complex assembly on the membrane was found; a  $\gamma$ M23K/ $\gamma$ R242E double mutant cannot correct the energy uncoupling situation [9]. In the present study, we will investigate the interaction between  $\gamma$ C87 and  $\gamma$ R242 and its effect on modulation of energy transmission in ATP synthase.

### 2. Materials and Methods

#### 2.1 Strains

*E. coli* strain DH5a (New England BioLabs) was used for mutagenesis [13]. Strain DK8 (*glnV44, rfbC1, endA1, spoT1, hfrPO1, bglR, thi-1, relA1, (atpB-atpC) ilv::Tn10,* Tetracycline resistant) was engineered by removing the *atp* (*unc*) operon from the chromosome of *E. coli* strain 1100 to yield a background without intrinsic ATP synthase [14,15].

#### 2.2 Construction of plasmids

The pSN6 plasmid (pBR322 derivative, *atpB-atpC*, Ampicillin resistant) was used as the wild type (WT) throughout this study [16]. Oligonucleotides (Integrated DNA Technology, listed in Supplementary Material Table S2) for site-directed mutagenesis were applied in the polymerase chain reaction (PCR) by following the recommended protocol (Q5® High Fidelity DNA Polymerase, New England BioLabs). Presence of the desired mutation was verified by DNA sequencing, followed by transformation into strain DK8.

#### 2.3 Growth yield assay

A single colony of DK8 strain harboring WT or mutant ATP synthase was inoculated in lysogeny broth (LB) medium with 100  $\mu$ g/mL ampicillin and grown to late exponential phase. A further 1:500 inoculation was made into medium containing 8 mM succinate, allowing it to grow aerobically until saturation at 30 °C or 37 °C [17]. Growth yield was evaluated from turbidity of the liquid culture by measuring its absorbance at 590 nm. Each mutant was assayed at least in triplicate.

#### 2.4 Inverted membrane vesicle preparations and protein concentration assay

Cells were pelleted at late exponential phase from aerobic growth at 30 °C. Resuspended cells were lysed by passage through a homogenizer. Membranes were then washed and pelleted as described [18]. For each strain expressing WT or mutant ATP synthase, at least two membrane vesicle preparations were made. Protein concentrations were determined by the Bradford method with bovine serum albumin as standard [19].

#### 2.5 Western Blot

The relative expression level of ATP synthase on membrane was determined by Western Blot with anti- $\beta$  antibody (Agrisera, Vannas, Sweden) or anti- $\gamma$  antibody (a kind gift from Drs. Toshiharu Suzuki and Masasuke Yoshida, Japan Science and Technology Agency, Tokyo) following standard protocols [20]. Band intensity was assessed by ImageJ software (National Institutes of Health). At least two biological replicate samples were quantified to minimize error.

#### 2.6 Functional analysis of ATP synthase

ATPase activities were assayed in a cocktail buffer containing 50 mM Tris/H<sub>2</sub>SO<sub>4</sub>, 4 mM MgSO<sub>4</sub>, 10 mM ATP and 1  $\mu$ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), pH 8.0, at 25 °C, 27 °C, 30 °C, 34 °C or 37 °C. To trigger the reaction, inverted membranes (10

 $\mu$ g/mL) were added into the cocktail buffer. To terminate the reaction, sodium dodecyl sulfate (SDS) was added to a final concentration of 5 % (w/v). Inorganic phosphate released from the reaction was quantified as described [21]. Each membrane sample was assayed at least in duplicate. To test the membrane proton pumping ability, 50 µg/mL of inverted membrane vesicles were suspended under vigorous stirring in 2.0 mL proton pumping buffer containing 10 mM HEPES, 300 mM KCl, 5 mM MgCl<sub>2</sub>, 1 µg/mL valinomycin and 1 µM acridine orange, pH 7.5, at 25 °C. Using an excitation wavelength of 460 nm, the acridine orange fluorescence intensity was measured at an emission wavelength of 530 nm. 1 mM ATP or 2 mM NADH was added to initiate proton pumping; 5 µM CCCP was added to dissipate the proton gradient [22]. Each sample was measured at least in duplicate.

#### 2.7 Transition state thermodynamic parameters

The apparent enzyme activation energy for ATP hydrolysis by ATP synthase was obtained by measuring turnover number as a function of the temperature terms as given in the Arrhenius equation [23]. Turnover numbers were calculated by normalizing ATPase activities by the amount of membrane-bound ATP synthase determined by the Western Blot. Thermodynamic parameters can be resolved from the following equations:

$$\left[\frac{\partial \ln k_{cat}}{\partial (1/T)}\right]_p = -\frac{E_a}{R}$$

$$k_{cat} = Ae^{-\frac{E_{a}}{RT}}$$

$$E_a = \Delta H^{\ddagger} + RT$$

$$\Delta S^{\ddagger} = R \ln \left( \frac{AN_A h}{RT} \right) - R$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T \Delta S^{\ddagger}$$

In these equations, *R*, *A*,  $N_A$  and *h* represent the ideal gas constant, Arrhenius constant, Avogadro's number and Planck constant respectively.  $k_{cat}$  is the turnover number; *T*, the absolute temperature,  $E_a$ , the Arrhenius activation energy, and *G*, *H*, *S* are Gibbs free energy, enthalpy and entropy terms [24].

#### 2.8 Computational methods

Protein Data Bank (PDB) entry 3OAA (PDB ID: 3OAA) was adopted *in silico* as the parent protein model [25]. Chimera (University of California, San Francisco) software [26] was

used to visualize the molecular structure of ATP synthase. Computational study of electrostatics in ATP synthase was conducted in Python 2.7 with AESOP (Analysis of Electrostatic Similarities of Proteins) module [27,28]. Directed mutagenesis scan function was programed with its default parameters to calculate the Gibbs free energy of subunit association ( $G_a$ ) in ATP synthase [29]. To introduce a mutation in the original structure, the rotamers function was used in Chimera, followed by energy minimizing to eliminate all clashes among residues using the default settings [30].

All-atom molecular dynamics (MD) simulations with explicit solvents were performed in GROMACS [31] with an AMBER99sb force field [32] for the WT,  $\gamma$ C87K and  $\gamma$ C87K/  $\gamma$ R242Q mutants. Each protein was solvated in a cubic box with the simple point charge (SPC) water model and kept at least 10.0 A from borders [33]. Na<sup>+</sup> and Cl<sup>-</sup> ions were added into the system to neutralize charges in proteins at a concentration of 150 mM. The long-range electrostatic interactions among ions and charges in proteins were solved with the Particle Mesh Eward (PME) method [34]. The solvated systems were optimized until the largest force component was less than 10 kJ/(mol·nm) and equilibrated with the constrained protein conformations at 300 K and 1 atm over 200 ps. Unconstrained NPT MD simulations were numerically integrated for 5 ns at a time step of 2 fs, where trajectory information was saved using a 10 ps interval. The velocity rescaling thermostat method [35] and Parrinello-Rahman barostat method [36] were taken in equilibration steps and MD simulations.

#### 3. Results

#### 3.1 Mutation γC87K causes inefficient energy coupling in ATP synthase

Residue  $\gamma$ C87 is highly conserved; occasionally, an alanine is found in this position (Supplementary Material Table S1). Alanine (small hydrophobic), aspartate, glutamate (acidic) and phenylalanine (bulky hydrophobic) substitutions were made in the mutational study. To evaluate the performance of the WT or mutant ATP synthase, growth yield in succinate medium, enzyme expression level, ATPase activity and ATP-driven proton pumping ability were measured. The  $\gamma$ C87A/D/E mutants showed little adverse effect to the functions of ATP synthase, and they mirrored the WT in protein expression amount, ATPase activity and ATP-driven proton pumping ability. Strains carrying these mutations also maintained a substantial oxidative phosphorylation growth yield (> 80 % compared to the WT) in 8 mM succinate medium. The  $\gamma$ C87F mutant showed weaker ATP-driven proton pumping ability, as measured by acridine orange quenching, and lower growth yield (74 % and 73 % compared to the WT, respectively); however, it was still well capable to use nonfermentable carbon source efficiently.

Albeit  $\gamma$ C87 is not absolutely essential, ATP synthase with  $\gamma$ C87K mutation clearly presented a phenotype with poor utilization of succinate as the carbon source. Cells bearing such mutant enzyme hardly grew in succinate medium, resulting in a very low growth yield (< 20 % at 30 °C, <10 % at 37 °C) compared to the WT. Western Blot assay found that the amounts of ATP synthase assembled on the cell membrane were similar in the WT and the  $\gamma$ C87 mutants (Table 1). In addition, the *in vitro* ATPase activity assay and ATP-driven proton pumping assay illustrated that the  $\gamma$ C87K mutant was able to hydrolyze ATP with 60 % of the velocity as the WT, but it could barely convert the chemical energy released

from ATP hydrolysis to establish a transmembrane proton gradient (< 10 % ATP-driven proton pumping ability compared to the WT). The NADH-driven proton pumping assay clarified that the poor proton gradient observed with ATP was not because of passive leakage from damaged membrane vesicles. NADH-driven proton pumping by the  $\gamma$ C87K mutant gave acridine orange quenching signal of 75 % of WT; this slight reduction might be due to leakage through the uncoupled ATP synthase proton channel. All these observations indicated that the  $\gamma$ C87K mutation impaired the energy coupling between ATP hydrolysis/ synthesis and proton translocation across membrane in *E. coli* ATP synthase (Table 1). The  $\gamma$ C87K mutant does not abolish all functions, but it is able to support a small amount of oxidative phosphorylation *in vivo*. This result provides evidence that there should not be any severe structural or assembly defects in the holoenzyme since it still can utilize the transmembrane proton gradient (in contrast to the free F<sub>1</sub> complex of ATP synthase, which is completely uncoupled).

#### 3.2 Mutation $\gamma$ R242Q can suppress the energy uncoupling in the $\gamma$ C87K mutant

Residue  $\gamma$ R242 is located in a conserved region in the C-terminus of  $\gamma$  subunit; it appears strictly conserved (Supplementary Material Table S1). From the crystal structure of *E. coli* ATP synthase (PDB ID: 3OAA) [25], the  $\eta$ -N atom of  $\gamma$ R242 and the  $\gamma$ -S atom of  $\gamma$ C87are within 5 Å, so that an electrostatic repulsion might be caused by the  $\gamma$ C87K mutation. A random mutation at the  $\gamma$ R242 position was engineered by site-directed mutagenesis into the  $\gamma$ C87K mutant. DK8 cells harboring ATP synthase with double mutations were screened on 20 mM succinate agar medium, resulting in colonies of different sizes. Plasmids were purified from the large colonies for DNA sequencing. This mutagenesis strategy successfully discovered a second site revertant:  $\gamma$ R242Q (CAA). Compared to the  $\gamma$ C87K mutant (17 % at 30 °C, 8 % at 37 °C of WT), cells with the double mutations showed better growth yield (85 % at 30 °C, 90 % at 37 °C) in succinate medium. Moreover, the ATP-driven proton pumping strength was restored to 52 % compared to the WT (Table 2 and Fig. 1B).

#### 3.3 γR242A/C/S are weak suppressors of γC87K

To further explore the function of  $\gamma$ R242, alanine, cysteine, glutamate, leucine and serine replacements were engineered into the WT and the  $\gamma$ C87K mutant. The  $\gamma$ R242A/C/L/S mutants alone showed nearly WT-like growth yield, enzyme expression amount and enzymatic performance (Table 2 and Fig. 1A). In contrast, the  $\gamma$ R242E mutant showed lowered growth yield, especially under higher temperature (66 % at 30 °C, 30 % at 37 °C). A previous study indicated that the  $\gamma$ R242E mutation would interfere with ATP synthase assembly in cell membrane when *E. coli* cells were grown at 37 °C [9]. In the present study, the membranes were prepared from cells that grown at 30 °C, and ATP synthase was found assembled in the cell membranes by Western Blot and ATPase activity and proton pumping assay. Higher temperature appears to destabilize ATP synthase assembly in the  $\gamma$ R242E mutant.

Regarding to the restoration of energy coupling, a double mutant with  $\gamma$ C87K and  $\gamma$ R242A, C or S mutations could grow well in succinate medium at 30 °C (~ 70 % growth yield) but had worse performance at 37 °C (~ 30 % growth yield only).  $\gamma$ C87K/ $\gamma$ R242E or  $\gamma$ C87K/ $\gamma$ R242L double mutants had low growth yield (~ 30 % at 30 °C, < 10 % at 37 °C),

indicating that neither glutamate nor leucine in this position could effectively correct the energy uncoupling issue. The proton pumping ability for each mutant showed the similar trends as the growth yield (Table 2 and Fig. 1B).

#### 3.4 Mutations at YC87 and YR242 alter thermodynamic parameters of the transition state

In order to identify possible reasons for the inefficient energy coupling caused by the  $\gamma$ C87K mutation, thermodynamic parameters were analyzed by Arrhenius plots (Supplementary Material Fig. S2A and B). The thermodynamic properties of  $\gamma$ C87A/D/E/F mutations were very similar to those of WT; in sharp contrast, the YC87K enzyme showed an increased activation energy (WT + 19.4 kJ/mol). According to the transition state theory, this observation might indicate that extra interactions could occur between the  $\gamma$ C87K and adjacent residues. Thus, more energy is required to overcome a higher activation energy barrier to reach the transition state in the  $\gamma$ C87K mutant. If the raised activation energy could lead to the uncoupling phenotype of the  $\gamma$ C87K mutation, a secondary suppressor mutation at  $\gamma R242$  should correct the altered thermodynamic parameters. As shown in Table 3, all the  $\gamma$ R242A/C/Q/S mutants could somehow compensate the augmented activation energy in the  $\gamma$ C87K mutant and restore the energy coupling capability. The  $\gamma$ R242E mutant also showed a lowered activation energy, but it cannot well restore the efficient energy coupling because this glutamate replacement itself is found harmful to the function of ATP synthase. The  $\gamma$ C87K/ $\gamma$ R242L double mutant showed an even higher activation energy (WT + 32.7 kJ/mol) than the  $\gamma$ C87K mutant and it failed to reform the functional energy transmission.

#### 3.5 $\gamma$ C87 and $\gamma$ R242 residue may affect the subunit association

A mutagenesis analysis in silico was performed with the AESOP module to assess the influences of those  $\gamma$ C87 and  $\gamma$ R242 mutations. A single mutation was computationally engineered into ATP synthase once at a time. Through electrostatic stability evaluation among all the subunits ( $\alpha_3\beta_3\gamma\epsilon$  in PDB ID: 30AA), a result returned as the Gibbs free energy change of subunit association ( $G_a$ ) for the parent structure ( $G_a$  of WT = -21.5 kJ/ mol) and each of the mutant (Fig. 2). The  $\gamma$ C87K mutant presents a more negative  $G_a$ value compared to its parent structure (WT – 12.3 kJ/mol). The  $\gamma$ C87K mutation contributes to an overall electrostatic network and might make the ATP synthase too rigid to undergo conformational change smoothly. On the other hand,  $\gamma R242$  mutations weaken the overall electrostatic interaction to recover the flexibility in ATP synthase in the  $\gamma$ C87K double mutants. This is especially true to the  $\gamma$ R242E mutation, where the calculated  $G_a$  is greater than zero (+ 10.1 kJ/mol), indicating that, based purely on electrostatic interactions, subunit association should not be favored. This result fits to the observations and it might explain the relative instability of the mutant enzyme [9]. This computational approach reinforced our hypothesis that the  $\gamma$ C87K mutant would require higher energy to overcome the activation energy barrier and to achieve the transition state during nucleotide catalysis.

#### 3.6 Molecular dynamics simulations reveal possible residue interactions

The MD simulation the trajectories of WT,  $\gamma$ C87K and  $\gamma$ C87K/ $\gamma$ R242Q were visualized using Chimera. Fig. 3A, C and E show the residue conformation of the last frames from each MD simulation; distances among positions  $\beta_{\text{TP}}$ 381,  $\gamma$ C87,  $\gamma$ 238 and  $\gamma$ 242 are shown in Fig.

3B, D and F. Judged by enthalpy and protein backbone root-mean-square deviation (RMSD), equilibrium was reached after 2 ns. Hence, the unconstrained structures during the last 3 ns were used for residue interaction analysis. In the WT (Fig. 3A and B), the ensembleaveraged distance between  $\gamma$ C87 ( $\gamma$ -S) and  $\beta$ <sub>TP</sub>E381 (the nearer  $\epsilon$ -O) is 6.1  $\pm$  0.5 Å. The side chains of  $\gamma R242$  and  $\gamma E238$  form a hydrogen bond. In  $\gamma C87K$  (Fig. 3C and D), the hydrogen bond between  $\gamma R242$  and  $\gamma E238$  remains, and an additional hydrogen bond is formed between  $\gamma C87K$  and  $\beta_{TP}E381$ . The ensemble-averaged distance between  $\gamma C87K$  ( $\zeta$ -N) and  $\beta_{TP}E381$  (the nearer  $\epsilon$ -O) is 2.8 ± 0.1 Å. In the  $\gamma$ C87K/ $\gamma$ R242Q double mutant (Fig. 3E and F), hydrogen bonds between  $\gamma$ C87K and  $\beta_{TP}$ E381 and between  $\gamma$ R242 and  $\gamma$ E238 are no longer present; a new hydrogen bond is formed between  $\gamma$ C87K and  $\gamma$ R242Q. In this conformation, the ensemble-averaged distance between  $\gamma C87K$  ( $\zeta$ -N) and  $\beta_{TP}E381$  (the nearer  $\epsilon$ -O) is 7.5  $\pm$  0.4 Å. Compared with the inter-residue distances in the last 3 ns simulation (Fig. 3B, D and F), the distances in the WT show stronger fluctuations than those in the  $\gamma C87K/\gamma R242Q$  double mutant, and residues in the  $\gamma C87K$  mutant are even less mobile than in the double mutant. This observation indicates that residues at this  $\beta/\gamma$ interface would be more rigid in  $\gamma$ C87K than in the WT, and that the  $\gamma$ C87K/ $\gamma$ R242Q double mutations would allow a higher degree of flexibility to restore proper energy transmission.

#### 4. Discussion

#### 4.1 Energy transmission in ATP synthase

The long-term goal of this project is to illustrate the energy transmission mechanism in ATP synthase; our current target is the interface between the catalytic hexamer ( $\alpha_3\beta_3$ ) and the  $\gamma$  subunit. Two areas have been identified as responsible for the energy transmission: one is located between the N- and C-terminal helices of  $\gamma$  and the upper  $\beta$  catch loop; the other one is located between  $\gamma$  "neck" cluster and the  $\beta^{380}$ DELSEED<sup>386</sup> motif (the lower catch loop) [37]. Regarding the former area, previous studies have suggested that a network of hydrogen bonds and salt bridges is essential for communication between upper  $\beta$  catch loop and the coiled-coil helices of  $\gamma$  in *E. coli* ATP synthase, such as  $\gamma$ S12 and  $\beta$ D372,  $\gamma$ R268 and  $\beta$ D302/ $\beta$ D305 [10,38]. According to our recent study based on *Geobacillus stearothermophilus* ATP synthase, not individual amino acid side chain(s), but the overall coiled-coil shape of the N- and C-terminal helices of  $\gamma$  is the key feature to maintain functional energy transmission [39]. In the latter area, the  $\beta$ DELSEED motif has been widely studied for its importance in the energy relay and torque generation [40–43].

#### 4.2 Which part of ATP synthase would interact with γC87K?

Although the residue at  $\gamma 87$  is highly conserved among many species, it tolerates a mutation with small, bulky nonpolar or negatively charged side chain. The  $\gamma C87K$  mutant fails to maintain the proper energy transmission and thus perturbs the coupling between proton translocation and ATP synthesis/hydrolysis. Both the transition state thermodynamic analysis and subunit association calculations strongly suggest that extra electrostatic interactions could form upon the replacement of cysteine by lysine. The positive charge on lysine could form a salt bridge or hydrogen-bond with adjacent residues. The  $\beta DELSEED$  motif is rich of negative charges. The  $\beta DELSEED$  sequence is greatly conserved among

species (with only slight variations), but none of those five acidic residues is absolutely essential [20]. Many pieces of evidence support that the  $\beta$ DELSEED loop is involved in energy transmission through its conformational change upon nucleotide binding and release [43,44]. From the crystal structure of *E. coli* ATP synthase (PDB ID: 3OAA),  $\gamma$ C87 ( $\gamma$ -S) is 6.1 A away from  $\beta_{TP}E381$  ( $\epsilon$ -O); this distance would be even shorter upon the lysine replacement. Previous studies have reported that disulfide bonds form quickly between  $\gamma$ C87 and  $\beta$ E381C as well as  $\gamma$ C87 and  $\beta$ D380C under oxidizing conditions. As the covalent bond blocks rotation, ATP synthase abolishes both ATPase and proton pumping abilities [45,46]. The MD simulations reinforced our hypothesis. As shown in Fig. 3C, the bulky side chain of  $\gamma$ R242 ( $\eta$ -N) might bond with  $\gamma$ E238 ( $\epsilon$ -O1) (2.7 Å), prohibiting the ionic interaction between  $\gamma$ C87K ( $\zeta$ -N) with  $\gamma$ E238 ( $\epsilon$ -O1) (6.0 Å). An additional salt bridge or hydrogen bond formed between  $\gamma$ C87K ( $\zeta$ -N) and  $\beta_{TP}$ DELSEED (2.9 Å to the  $\epsilon$ -O of  $\beta_{TP}$ E381) could interfere with the normal rotation of ATP synthase.

# 4.3 How can a hydrogen bond between $\gamma$ C87K and $\beta_{TP}$ DELSEED disturb the energy coupling?

ATP synthesis and release rely on the sequential conformational alternation of three  $\beta$  subunits, which is driven by the torque from the rotor complex ( $c_{10}\gamma\epsilon$ ) [47]. However, in *E. coli* ATP synthase, the rotation of  $\gamma$  and of the c-ring are not well matched. The  $\gamma$  subunit undergoes one revolution in three successive 120° steps, whereas the c-ring steps by ten 36° progressions [48]; consequently, protons pushing the c-ring produce a torque stored in the rotor complex due to its elasticity [49]. Coupled by this elastic torsion, the torque observed in the rotor complex changes little despite of its rotation angle [50]. Smooth torque transmission from the rotor to the catalytic hexamer enables high efficiency and optimal performance of ATP synthase [51].

When an extra hydrogen bond is formed between  $\gamma$ C87K and  $\beta_{TP}E381$ , it drags the spin of the  $\gamma$  "neck" area. On the one hand, back slipping might occur when the torque generated from proton translocation is insufficient to overcome the elevated activation energy necessary for catalysis [52]. On the other hand, given the spin of  $\gamma$  "neck" motif is retarded while protons keep pushing the c-ring, the elastic energy built up within the rotor complex would be larger than in absence of the extra hydrogen bond [53]. When the elastic tension accumulated finally becomes large enough to break the hydrogen bond, the energy is released abruptly, with  $\gamma$  at more advanced rotational angle than usual. Such "jumpy" rotation of  $\gamma$  with a later release point of  $\beta$ - $\gamma$  interactions would impact the energy transmission from the rotor complex to the catalytic hexamer and the subsequent conformational alternation of the  $\beta$  subunits. Neither back slipping nor "jumpy" spin of  $\gamma$ gives smooth and effective conformational change in the  $\beta$  subunits; hence, less ATP will be synthesized per time. In addition, considering the enzyme is not absolutely elastic, energy from proton gradient is consumed during unnecessary conformational change in ATP synthase, so that the energy coupling rate (ATP synthesis per proton) may become lower. This hypothesis can also explain the absence of an effect when  $\gamma C87$  is replaced by a neutral or acidic amino acid.

# 4.4 Efficient energy coupling in ATP synthase relies on a cluster consisting of $\gamma$ C87, $\gamma$ M23 and $\beta_{TP/dp}$ E381

Previous studies have documented that the  $\gamma$ M23K/R [22] and  $\beta$ E381K/R mutations [9,54] impair energy coupling between proton translocation and ATP synthesis/hydrolysis. Comparison with the results on the  $\gamma$ C87K mutation from the present study shows similarities between these uncoupling mutations. First, they all have a side chain with a positive charge; second, they are spatially close to each other with potential interactions; third, they are all located at the  $\beta$ DELSEED/ $\gamma$ -neck interface which is critical to maintain efficient energy coupling (Fig. 4). With other types of mutation such as  $\gamma$ M23D/E/L [4],  $\gamma$ C87A/D/E/F (this study) and  $\beta$ E381A/D/Q [9], the functions of the enzyme are not affected. We propose that a high density of positive charge in this area prevents proper energy transmission in ATP synthase.

#### 4.5 Intra-subunit communication between γC87 and γR242

The  $\gamma$ R242C mutation can suppress inefficient energy coupling caused by the  $\gamma$ C87K (this study) or the  $\gamma$ M23K mutation [12]. A previous study on the  $\gamma$ M23K/ $\gamma$ R242C double mutant proposed that the cysteine residue in the  $\gamma$ R242C mutation would remove the ionic bond between  $\gamma R242$  and  $\beta E381$  and would form a repulsive ion pair between the thiolate and  $\beta$ E381 [55]. In that way,  $\gamma$ R242C would be able to compensate the uncoupling effect of  $\gamma$ M23K. Can we adopt the same idea to explain the observations with the  $\gamma$ C87K mutation? First, it has to be pointed out that, although the  $\gamma$ C87K and  $\gamma$ M23K mutations share similarities in the thermodynamic patterns of the transition state,  $\gamma C87K$  more likely interacts with  $\beta_{TP}E381$  whereas the  $\gamma M23K$  interacts with  $\beta dpE381$  [9]. Viewed from the original crystal structure (PDB ID: 30AA) shown in Fig. 4, the distance between  $\gamma$ R242 ( $\eta$ -N) and  $\beta_{TP}E381$  (e-O) or  $\beta dpE381$  (e-O) residues is 8.2 Å and 14.8 Å respectively ( $\beta_e E381$ is > 20 Å away and is inaccessible due to blockage by the helical portion of the  $\gamma$  subunit); thus, the diminished interaction between the  $\gamma R242C$  mutant and  $\beta E381$  may not significantly affect the enzyme rigidity during catalysis. Second, the pKa value of a cysteine side chain is 8.5 (in water), and it requires a basic local environment to deprotonate the thiol into thiolate. Finally, except for  $\gamma$ R242C, the charge repulsion hypothesis cannot explain other mutations such as  $\gamma R242A/S/Q$ . Instead, most of the  $\gamma R242$  mutations are more likely to destabilize the interaction with  $\gamma E238$ . The electrostatic attraction between  $\gamma R242$  and  $\beta$ E381 is not essential to ATP synthase, since many  $\beta$ E381 mutants [9,54] and  $\gamma$ R242 mutants (this study) are WT-like.

How do the  $\gamma$ R242 mutations interact with  $\gamma$ C87K? If we accept the hypothesis that  $\gamma$ C87K forms extra bonds with the  $\beta$ DELSEED motif resulting in energy transmission deficiency (Fig. 3C), in order to restore the functional energy flow,  $\gamma$ R242 mutations should lead the  $\gamma$ C87K away from the  $\beta$ DELSEED motif. At 30 °C, in the  $\gamma$ R242A/C/S mutant, the less bulky side chain together with the removal of the positive charge seems to allow  $\gamma$ C87K to bend back toward the interior of  $\gamma$ . The MD simulations (Fig. 3E) suggest that, although the side chain of  $\gamma$ R242Q is not that small, its polar moiety can well hydrogen-bond to the lysine headgroup in  $\gamma$ C87K (the distance between  $\gamma$ C87K  $\zeta$ -N to  $\gamma$ R242Q  $\epsilon$ -O is 2.8 Å). Furthermore, small or polar substitutions of  $\gamma$ R242 could allow  $\gamma$ C87K to form a salt bridge with  $\gamma$ E238, thus stabilizing the lysine side chain ( $\zeta$ -N) away from  $\beta_{TP}$ E381 ( $\epsilon$ -O) (8.0 Å).

At 37 °C, only  $\gamma$ R242Q is able to correct the energy coupling issue satisfactorily. For side chains in position  $\gamma$ R242 with weaker or no interactions with the lysine in position  $\gamma$ 87 (such as  $\gamma$ R242A/C/S), the increasing molecular freedom would give  $\gamma$ C87K more flexibility to interact with the  $\beta$ DELSEED motif instead of  $\gamma$ E238.  $\gamma$ R242L can recover the energy coupling neither at 37 °C nor at 30 °C because its bulky and nonpolar side chain blocks  $\gamma$ C87K bonding with  $\gamma$ E238. The  $\gamma$ R242E mutant alone already results in poor ATP-driven proton pumping ability and low growth yield; it is uncapable to correct the energy uncoupling issue in the ATP synthase with  $\gamma$ C87K/ $\gamma$ R242E double mutations either.

To summarize,  $\gamma R242$  has limited direct interaction with  $\beta_{TP}E381$  but the residue in this position modulates the orientation of  $\gamma C87K$  and affects its interaction with  $\gamma E238$ . This discovery supports the notion that the residue communications at the  $\beta/\gamma$  interface are of crucial importance for energy transmission in ATP synthase.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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#### Abbreviations footnote

AMP-PNP	adenylyl-imidodiphosphate
СССР	carbonyl cyanide <i>m</i> -chlorophenylhydrazone
MD	molecular dynamics

#### References

- [1]. Sobti M, Smits C, Wong AS, Ishmukhametov R, Stock D, Sandin S, Stewart AG, Cryo-EM structures of the autoinhibited E. coli ATP synthase in three rotational states, Elife. 5 (2016).
- [2]. Weber J, Structural biology: Toward the ATP synthase mechanism, Nat. Chem. Biol 6 (2010) 794– 795. [PubMed: 20956969]
- [3]. Stock D, Leslie AG, Walker JE, Molecular architecture of the rotary motor in ATP synthase, Science. 286 (1999) 1700–1705. [PubMed: 10576729]
- [4]. Shin K, Nakamoto RK, Maeda M, Futai M, F0F1-ATPase gamma subunit mutations perturb the coupling between catalysis and transport, J. Biol. Chem 267 (1992) 20835–20839. [PubMed: 1400398]
- [5]. Omote H, Sambonmatsu N, Saito K, Sambongi Y, Iwamoto-Kihara A, Yanagida T, Wada Y, Futai M, The gamma-subunit rotation and torque generation in F1-ATPase from wild-type or

uncoupled mutant *Escherichia coli*, Proc. Natl. Acad. Sci. U. S. A 96 (1999) 7780–7784. [PubMed: 10393898]

- [6]. Hausrath AC, Capaldi RA, Matthews BW, The conformation of the epsilon- and gammasubunits within the *Escherichia* coli F(1) ATPase, J. Biol. Chem 276 (2001) 47227–47232. [PubMed: 11585832]
- [7]. Weber J, Senior AE, ATP synthesis driven by proton transport in F1F0-ATP synthase, FEBS Lett. 545 (2003) 61–70. [PubMed: 12788493]
- [8]. Caviston TL, Ketchum CJ, Sorgen PL, Nakamoto RK, Cain BD, Identification of an uncoupling mutation affecting the b subunit of F1F0 ATP synthase in *Escherichia coli*, FEBS Lett. 429 (1998) 201–206. [PubMed: 9650590]
- [9]. Ketchum CJ, Al-Shawi MK, Nakamoto RK, Intergenic suppression of the gammaM23K uncoupling mutation in F0F1 ATP synthase by betaGlu-381 substitutions: the role of the beta380DELSEED386 segment in energy coupling, Biochem. J 330 (Pt 2) (1998) 707–712. [PubMed: 9480879]
- [10]. Lowry DS, Frasch WD, Interactions between βD372 and γ Subunit N-Terminus Residues γK9 and γS12 Are Important to Catalytic Activity Catalyzed by *Escherichia coli* F1Fo-ATP Synthase, Biochemistry. 44 (2005) 7275–7281. [PubMed: 15882066]
- [11]. Buchert F, Schober Y, Rompp A, Richter ML, Forreiter C, Reactive oxygen species affect ATP hydrolysis by targeting a highly conserved amino acid cluster in the thylakoid ATP synthase gamma subunit, Biochim. Biophys. Acta 1817 (2012) 2038–2048. [PubMed: 22727877]
- [12]. Nakamoto RK, Maeda M, Futai M, The gamma subunit of the *Escherichia coli* ATP synthase. Mutations in the carboxyl-terminal region restore energy coupling to the amino-terminal mutant gamma Met-23-->Lys, J. Biol. Chem 268 (1993) 867–872. [PubMed: 8419364]
- [13]. Anton BP, Raleigh EA, Complete Genome Sequence of NEB 5-alpha, a Derivative of Escherichia coli K-12 DH5alpha, Genome Announc. 4 (2016).
- [14]. Klionsky DJ, Brusilow WS, Simoni RD, In vivo evidence for the role of the epsilon subunit as an inhibitor of the proton-translocating ATPase of *Escherichia coli*, J. Bacteriol 160 (1984) 1055– 1060. [PubMed: 6238948]
- [15]. Bachmann BJ, Pedigrees of some mutant strains of Escherichia coli K-12, Bacteriol. Rev 36 (1972) 525–557. [PubMed: 4568763]
- [16]. Mao HZ, Abraham CG, Krishnakumar AM, Weber J, A functionally important hydrogen-bonding network at the betaDP/alphaDP interface of ATP synthase, J. Biol. Chem 283 (2008) 24781– 24788. [PubMed: 18579516]
- [17]. Weber J, Bowman C, Wilke-Mounts S, Senior AE, alpha-Aspartate 261 is a key residue in noncatalytic sites of *Escherichia coli* F1-ATPase, J. Biol. Chem 270 (1995) 21045–21049.
   [PubMed: 7673131]
- [18]. Gajadeera CS, Weber J, *Escherichia coli* F1Fo-ATP synthase with a b/delta fusion protein allows analysis of the function of the individual b subunits, J. Biol. Chem 288 (2013) 26441–26447. [PubMed: 23893411]
- [19]. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem 72 (1976) 248–254.
   [PubMed: 942051]
- [20]. Mnatsakanyan N, Kemboi SK, Salas J, Weber J, The beta subunit loop that couples catalysis and rotation in ATP synthase has a critical length, J. Biol. Chem 286 (2011) 29788–29796. [PubMed: 21705326]
- [21]. Taussky HH, Shorr E, A simplified method for estimating urinary inorganic phosphate during aluminum gel therapy for phosphatic calculi, J. Urol 69 (1953) 454–455. [PubMed: 13035938]
- [22]. Ketchum CJ, Nakamoto RK, A mutation in the *Escherichia coli* F0F1-ATP synthase rotor, gammaE208K, perturbs conformational coupling between transport and catalysis, J. Biol. Chem 273 (1998)22292–22297. [PubMed: 9712846]
- [23]. Al-Shawi MK, Ketchum CJ, Nakamoto RK, Energy coupling, turnover, and stability of the F0F1 ATP synthase are dependent on the energy of interaction between gamma and beta subunits, J. Biol. Chem 272 (1997) 2300–2306. [PubMed: 8999937]

- [24]. Al-Shawi MK, Senior AE, Complete kinetic and thermodynamic characterization of the unisite catalytic pathway of *Escherichia coli* F1-ATPase. Comparison with mitochondrial F1-ATPase and application to the study of mutant enzymes, J. Biol. Chem 263 (1988) 19640–19648. [PubMed: 2904441]
- [25]. Cingolani G, Duncan TM, Structure of the ATP synthase catalytic complex F(1) from Escherichia coli in an autoinhibited conformation, Nat. Struct. Mol. Biol 18 (2011) 701–707. [PubMed: 21602818]
- [26]. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE, UCSF chimera - A visualization system for exploratory research and analysis, J. Comput. Chem 25 (2004) 1605–1612. [PubMed: 15264254]
- [27]. Kieslich CA, Gorham RD, Morikis D, Is the rigid-body assumption reasonable? Insights into the effects of dynamics on the electrostatic analysis of barnase-barstar, J. Non. Cryst. Solids 357 (2011)707–716.
- [28]. Gorham RD, Kieslich CA, Nichols A, Sausman NU, Foronda M, Morikis D, An Evaluation of Poisson-Boltzmann Electrostatic Free Energy Calculations through Comparison with Experimental Mutagenesis Data, Biopolymers. 95 (2011) 746–754. [PubMed: 21538330]
- [29]. Harrison RES, Mohan RR, Gorham RD Jr., Kieslich CA, Morikis D, AESOP: A Python Library for Investigating Electrostatics in Protein Interactions, Biophys. J 112 (2017) 1761–1766. [PubMed: 28494947]
- [30]. Shapovalov MV, Dunbrack RL, A Smoothed Backbone-Dependent Rotamer Library for Proteins Derived from Adaptive Kernel Density Estimates and Regressions, Structure. 19 (2011) 844–858. [PubMed: 21645855]
- [31]. Pall S, Abraham MJ, Kutzner C, Hess B, Lindahl E, Tackling exascale software challenges in molecular dynamics simulations with GROMACS In: Solving Software Challenges for Exascale. EASC 2014. Lecture Notes in Computer Science, in: Solving Softw. Challenges Exascale, 2015.
- [32]. Hornak V, Abel R, Okur A, Strockbine B, Roitberg A, Simmerling C, Comparison of multiple amber force fields and development of improved protein backbone parameters, Proteins Struct. Funct. Genet (2006).
- [33]. Van Der Spoel D, Van Maaren PJ, Berendsen HJC, A systematic study of water models for molecular simulation: Derivation of water models optimized for use with a reaction field, J. Chem. Phys (1998).
- [34]. Darden T, York D, Pedersen L, Particle mesh Ewald: An N log(N) method for Ewald sums in large systems, J. Chem. Phys (1993).
- [35]. Bussi G, Donadio D, Parrinello M, Canonical sampling through velocity rescaling, J. Chem. Phys (2007).
- [36]. Marto ák R, Laio A, Parrinello M, Predicting Crystal Structures: The Parrinello-Rahman Method Revisited, Phys. Rev. Lett (2003).
- [37]. Pu J, Karplus M, How subunit coupling produces the gamma-subunit rotary motion in F1-ATPase, Proc. Natl. Acad. Sci. U. S. A 105 (2008) 1192–1197. [PubMed: 18216260]
- [38]. Greene MD, Frasch WD, Interactions among γR268, γQ269, and the β Subunit Catch Loop of Escherichia coli F1-ATPase Are Important for Catalytic Activity, J. Biol. Chem (2003).
- [39]. Mnatsakanyan N, Li Y, Weber J, Identification of two segments of the subunit of ATP synthase responsible for the different affinities of the catalytic nucleotide-binding sites, J. Biol. Chem 294 (2019).
- [40]. Hara KY, Noji H, Bald D, Yasuda R, Kinosita K Jr., Yoshida M, The role of the DELSEED motif of the beta subunit in rotation of F1-ATPase, J. Biol. Chem 275 (2000) 14260–14263. [PubMed: 10799504]
- [41]. Tanigawara M, Tabata KV, Ito Y, Ito J, Watanabe R, Ueno H, Ikeguchi M, Noji H, Role of the DELSEED loop in torque transmission of F1-ATPase, Biophys. J 103 (2012) 970–978. [PubMed: 23009846]
- [42]. La T, Clark-Walker GD, Wang X, Wilkens S, Chen XJ, Mutations on the N-terminal edge of the DELSEED loop in either the alpha or beta subunit of the mitochondrial F1-ATPase enhance ATP hydrolysis in the absence of the central gamma rotor, Eukaryot. Cell 12 (2013) 1451–1461. [PubMed: 24014764]

- [43]. Watanabe R, Koyasu K, You H, Tanigawara M, Noji H, Torque transmission mechanism via DELSEED loop of F1-ATPase, Biophys. J 108 (2015) 1144–1152. [PubMed: 25762326]
- [44]. Gledhill JR, Montgomery MG, Leslie AG, Walker JE, How the regulatory protein, IF(1), inhibits F(1)-ATPase from bovine mitochondria, Proc. Natl. Acad. Sci. U. S. A 104 (2007) 15671–15676.
  [PubMed: 17895376]
- [45]. Zhou Y, Duncan TM, Cross RL, Subunit rotation in *Escherichia coli* FoF1-ATP synthase during oxidative phosphorylation, Proc. Natl. Acad. Sci. U. S. A 94 (1997) 10583–10587. [PubMed: 9380678]
- [46]. Feng Z, Aggeler R, Haughton MA, Capaldi RA, Conformational changes in the Escherichia coli ATP synthase (ECF1F0) monitored by nucleotide-dependent differences in the reactivity of Cys-87 of the gamma subunit in the mutant betaGlu-381 --> Ala, J. Biol. Chem 271 (1996) 17986–17989. [PubMed: 8663500]
- [47]. Czub J, Grubmuller H, Torsional elasticity and energetics of F1-ATPase, Proc. Natl. Acad. Sci. U. S. A 108 (2011) 7408–7413. [PubMed: 21502534]
- [48]. Sielaff H, Rennekamp H, Wachter A, Xie H, Hilbers F, Feldbauer K, Dunn SD, Engelbrecht S, Junge W, Domain compliance and elastic power transmission in rotary FOF1-ATPase, Proc. Natl. Acad. Sci (2008).
- [49]. Martin JL, Ishmukhametov R, Spetzler D, Hornung T, Frasch WD, Elastic coupling power stroke mechanism of the F1-ATPase molecular motor, Proc. Natl. Acad. Sci. U. S. A 115 (2018) 5750– 5755. [PubMed: 29760063]
- [50]. Panke O, Cherepanov DA, Gumbiowski K, Engelbrecht S, Junge W, Viscoelastic dynamics of actin filaments coupled to rotary F-ATPase: angular torque profile of the enzyme, Biophys. J 81 (2001) 1220–1233. [PubMed: 11509339]
- [51]. Junge W, Sielaff H, Engelbrecht S, Torque generation and elastic power transmission in the rotary F(O)F(1)-ATPase, Nature. 459 (2009) 364–370. [PubMed: 19458712]
- [52]. Sekiya M, Nakamoto RK, Al-Shawi MK, Nakanishi-Matsui M, Futai M, Temperature dependence of single molecule rotation of the *Escherichia coli* ATP synthase F1 sector reveals the importance of gamma-beta subunit interactions in the catalytic dwell, J. Biol. Chem 284 (2009)22401–22410. [PubMed: 19502237]
- [53]. Cherepanov DA, Mulkidjanian AY, Junge W, Transient accumulation of elastic energy in proton translocating ATP synthase, FEBS Lett. 449 (1999) 1–6. [PubMed: 10225416]
- [54]. Azim S, Ahmad Z, Glu residues of betaDELSEED-motif are essential for peptide binding in *Escherichia coli* ATP synthase, Int. J. Biol. Macromol 116 (2018) 977–982. [PubMed: 29782980]
- [55]. Al-Shawi MK, Nakamoto RK, Mechanism of energy coupling in the FOF1-ATP synthase: the uncoupling mutation, gammaM23K, disrupts the use of binding energy to drive catalysis, Biochemistry. 36 (1997) 12954–12960. [PubMed: 9335555]

## Highlights

• The  $\gamma$ C87K mutation impairs energy transmission in *E. coli* ATP synthase.

- Extra hydrogen bond forms between  $\gamma$ C87K and the  $\beta_{TP}$ DELSEED loop.
- An additional  $\gamma$ R242Q/S/C/A mutation can restore the energy transmission.
- The  $\gamma$ R242E/L mutation cannot restore the energy transmission.
- The  $\gamma$ R242Q is most effective to suppress the energy uncoupling.

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#### Fig. 1.

Proton gradient formation ability of WT or mutant ATP synthase. The quenching of the fluorescence signal reflects the establishment of a proton gradient. ATP-driven proton pumping assay conditions were the same as described in the legend of Table 1. 1 mM ATP was added in the proton pumping buffer at Time = 40 s to initiate the proton translocation; 5  $\mu$ M CCCP was added at Time = 385 s. (A) Proton pumping ability of  $\gamma$ R242 mutants. (B) Proton pumping ability of  $\gamma$ C87K with additional  $\gamma$ R242 mutants.

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#### Fig. 2.

Gibbs free energies of subunit association. Mutations were introduced *in silico* based on the structure of PDB ID: 3OAA. The overall electrostatic stability was calculated through the directed mutagenesis scan function in the AESOP module. This method suggests a thermal fluctuation with  $kT \sim \pm 2.5$  kJ/mol [29]. The Gibbs free energy of subunit association for each mutant was normalized against the WT (– 21.5 kJ/mol). A negative value of free energy of association in this figure indicates that the electrostatic interaction becomes stronger in the mutant ATP synthase, and it turns the enzyme into a more rigid complex compared to the parent structure.





MD Simulation Steps (10 ps per step)



#### Fig. 3.

Interactions of  $\gamma$ C87 with the  $\beta_{TP}$ DELSEED loop. WT ATP synthase as well as  $\gamma$ C87K and  $\gamma$ C87K/ $\gamma$ R242Q mutant structures were loaded into GROMACS for MD analysis. After 5 ns simulation, the last frame of each structure was illustrated by Chimera. In the Figures A, C and E,  $\beta_{TP}$  and  $\gamma$  subunits are colored yellow and cyan respectively, and atoms are distinguished by CPK color mode (carbon element in gray, nitrogen in blue and oxygen in red). The distances between a pair of atoms are shown in Å. Figures B, D and F show the distances between selected atoms *versus* the steps during MD simulations (10 ps per step).

(A) WT ATP synthase. The distance between  $\beta_{TP}E381$  (e-O) and  $\gamma C87$  ( $\gamma$ -S) is 6.1 Å. (B) MD distances between atoms in WT. Red:  $\gamma$ E238 (e-O) and  $\gamma$ R242 ( $\eta$ -N), Magenta:  $\beta_{TP}E381$  (e-O) and  $\gamma R242$  ( $\eta$ -N), Blue:  $\beta_{TP}E381$  (e-O) and  $\gamma C87$  ( $\gamma$ -S), Black:  $\gamma C87$  ( $\gamma$ -S) and  $\gamma$ E238 ( $\epsilon$ -O), Green:  $\gamma$ C87 ( $\gamma$ -S) and  $\gamma$ R242 ( $\eta$ -N). (C)  $\gamma$ C87K ATP synthase. Due to electrostatic repulsion and spatial hindrance by  $\gamma R242$ , the  $\gamma C87K$  side chain folds toward  $\beta_{\text{TP}}$ E381. This increased  $\beta/\gamma$  interaction may restrict ATP synthase from normal rotation. The distance between  $\beta_{TP}E381$  (e-O) and  $\gamma C87K$  ( $\zeta$ -N) is 2.9 Å. (**D**) MD distances between atoms in the  $\gamma$ C87K mutant. Red:  $\gamma$ E238 ( $\epsilon$ -O) and  $\gamma$ R242 ( $\eta$ -N), Magenta:  $\beta$ <sub>TP</sub>E381 ( $\epsilon$ -O) and  $\gamma R242$  ( $\eta$ -N), Blue:  $\beta_{TP}E381$  (e-O) and  $\gamma C87K$  ( $\zeta$ -N), Black:  $\gamma C87K$  ( $\eta$ -N) and  $\gamma E238$ ( $\epsilon$ -O), Green:  $\gamma$ C87K ( $\eta$ -N) and  $\gamma$ R242 ( $\eta$ -N). (E)  $\gamma$ C87K/ $\gamma$ R242Q ATP synthase. Weakened charge repulsion from  $\gamma R242Q$  allows the  $\gamma C87K$  side chain to bond with  $\gamma E238$ and  $\gamma R242Q$  itself. Moreover, the polar moiety of glutamine may further coordinate the  $\gamma$ C87K side chain away from the  $\beta_{TP}$ DELSEED motif. The diminished interaction between  $\gamma$ C87K and  $\beta$ <sub>TP</sub>DELSEED rescues ATP synthase from inefficient energy coupling. In this model, the distance between  $\beta_{TP}E381$  (e-O) and  $\gamma C87K$  ( $\zeta$ -N) is 8.0 Å. (F) MD distances between atoms in the  $\gamma$ C87K/ $\gamma$ R242Q mutant. Red:  $\gamma$ E238 ( $\epsilon$ -O) and  $\gamma$ R242Q ( $\epsilon$ -O), Blue:  $\beta_{TP}E381$  (e-O) and  $\gamma C87K$  ( $\zeta$ -N), Black:  $\gamma C87K$  ( $\zeta$ -N) and  $\gamma E238$  (e-O), Green:  $\gamma C87K$  $(\zeta$ -N) and  $\gamma$ R242Q ( $\epsilon$ -O).

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#### Fig. 4.

Spatial relationship of residues located at the  $\beta$  and  $\gamma$ -neck interface. The figure is based on the crystal structure of *E. coli* ATP synthase (PDB ID: 3OAA).  $\beta_{TP}$ ,  $\beta_{DP}$  and  $\gamma$  subunits are colored yellow, orange and cyan respectively. The CPK color mode is applied to distinguish different elements (carbon in gray, nitrogen in blue and oxygen in red). Distances among selected atom pairs are shown in A. A residue labeled in blue shows that it has been reported (Refs. 9, 22, 54 or this study) to impair efficient energy coupling when replaced by lysine or arginine.

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Energy coupling properties of  $\gamma$ C87 mutants

Strain	Growth Yield	ATP Synthase Amount	ATPase Activity	ATP-driven H <sup>+</sup> Pumping	NADH-driven H <sup>+</sup> Pumping
	%	%	unit/mg protein	%	%
WΤ	100	100	4.6 (0.8)	100	100
rC87A	95 (2)	90 (10)	3.9 (0.2)	88 (5)	90 (5)
C87D	92 (3)	90 (10)	5.7 (0.3)	101 (3)	ND
<b>у</b> С87Е	84 (3)	130 (20)	7.5 (0.4)	92 (4)	ND
yC87F	74 (4)	90 (10)	5.1 (0.4)	73 (5)	ND
/C87K	17 (2)	80 (10)	2.8 (0.5)	6 (3)	75 (6)
oUC18	$^{<1}$	0	< 0.01	<1	99 (1)

d was quantified from the turbidity of cell culture by measuring wavelength at 460 nm. 5 µM CCCP was added to terminate the reaction and to establish 100 % fluorescence intensity. All percentage values in this table are normalized against WT. Standard deviations are absorbance at 590 nm. The membrane-bound ATP synthase amount was measured by Western Blot using anti-y antibody. ATPase activities were determined by the amount of inorganic phosphate released buffer. Either 1 mM ATP or 2 mM NADH was added to initiate proton pumping across membrane. Acridine orange fluorescence intensities were monitored at emission wavelength 530 nm with excitation shown in parenthesis. DK8 with pUC18 plasmid serves as a negative control, harboring no atp operon. ND, not determined; since these mutants showed WT-like ATP-driven proton pumping ability, their at 37 °C; 1 unit is defined as 1 µmol inorganic phosphate released per minute. To evaluate proton pumping ability of ATP synthase, inverted cell membrane vesicles were suspended in proton pumping membrane vesicles should maintain integrity.

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Strain	Growt	h Yield	ATP Synthase Amount	ATPase Activity	ATP-driven H <sup>+</sup> Pumping	NADH-driven H <sup>+</sup> Pumping
	30 °C %	37 °C %	%	unit/mg protein	%	%
WT	100	100	100	4.6 (0.8)	100	100
$\gamma$ C87K	17 (2)	8 (1)	80 (10)	2.8 (0.5)	6 (3)	75 (6)
γR242A	86 (4)	97 (3)	110(10)	4.9 (0.5)	76 (5)	84 (3)
yR242C	95 (3)	98 (4)	110 (20)	2.4 (0.4)	87 (4)	95 (3)
γR242E	66 (4)	30 (3)	100 (10)	2.3 (0.4)	48 (4)	94 (3)
γR242L	81 (2)	85 (4)	100 (10)	2.4 (0.2)	56 (5)	85 (4)
yR242S	99 (2)	97 (4)	100 (10)	2.5 (0.3)	88 (4)	97 (2)
7K/γR242A	75 (4)	23 (4)	110 (10)	4.0 (0.6)	30 (3)	84 (3)
7K/γR242C	72 (3)	35 (3)	110 (20)	3.1 (0.4)	32 (4)	85 (5)
7K/γR242E	33 (4)	8 (2)	70 (10)	1.6 (0.2)	8 (2)	90 (3)
7K/γR242L	31 (3)	7 (2)	80 (10)	1.7 (0.2)	3 (2)	92 (3)
7K/γR242Q	85 (4)	90 (5)	110(10)	4.5 (0.7)	52 (4)	80 (5)
7K/γR242S	73 (3)	22 (4)	110(10)	4.0 (0.5)	45 (5)	72 (6)

able are normalized against WT. Standard deviations are shown in parenthesis.

# Table 3

Transition state thermodynamic parameters of  $\gamma C87$  and  $\gamma R242$  mutants

Strain	H‡ kJ/mol	$T S^{\sharp}$ kJ/mol	G <sup>#</sup> kJ/mol
WT	33.6 (1.7) ( H <sup>‡</sup> ) kJ/mol	– 37.0 (1.7) ( <b>T</b> S <sup>‡</sup> ) kJ/mol	70.6 (0.0) ( G <sup>‡</sup> ) kJ/mol
$\gamma C87A$	- 2.9 (0.5)	- 2.9 (0.5)	0.0 (0.0)
$\gamma$ C87D	- 2.3 (1.0)	- 1.3 (1.0)	-1.0(0.0)
$\gamma C87E$	- 2.8 (0.3)	- 1.1 (0.3)	- 1.7 (0.0)
$\gamma C87F$	+2.9(0.6)	+ 3.6 (0.6)	-0.5(0.0)
$\gamma$ C87K	+ 19.4 (2.3)	+ 17.0 (2.2)	+ 2.4 (0.1)
رC87K/γR242A	+ 1.8 (2.1)	+ 1.4 (2.0)	+0.4(0.1)
C87K/γR242C	+ 12.8 (1.1)	+ 11.4 (1.1)	+1.4(0.0)
,C87K/γR242E	+ 12.2 (1.2)	+ 10.1 (1.2)	+ 2.1 (0.1)
,C87K/γR242L	+ 32.7 (0.4)	+ 30.1 (0.4)	+ 2.7 (0.0)
,C87К/γR242Q	+ 8.1 (0.9)	+ 7.8 (0.9)	+0.3(0.0)
rC87K/γR242S	+ 1.4 (1.0)	+ 1.1 (1.0)	+0.3(0.0)

ATPase activities of membrane-bound ATP synthase were measured at 25 °C, 27 °C, 30 °C, 34 °C and 37 °C. All terms were calculated for Arrhenius plots at 25 °C as described under Materials and Methods. values give the differences between mutant enzymes and the WT. Standard deviations are shown in parenthesis.